Improved Assay Procedure for Determination of Milk-Clotting Enzymes[1]

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ABSTRACT

An assay technique for quantifying milk-clotting enzymes is described. By the technique, enzyme concentrations as small as 2×10^{-5} rennet units/ml can be determined accurately in milk solutions in as little as 2 h. The technique can be used to assay for enzymes in milk and whey solutions without prior removal of casein. The technique should find application in detecting enzymes in a variety of systems commonly encountered in studies of milk and cheese products.

INTRODUCTION

Quantification of milk-clotting activity in solutions containing proteolytic enzymes is a major concern in industrial cheese making and cheese research. Many assay techniques for quantifying renneting activity have been described (1, 3, 4, 5, 9, 11, 14, 15, 19, 20, 22, 23, 24, 25, 28). Immunological agglutination reactions have been used to detect chymosin, porcine pepsin, and bovine pepsin in cheese and milk products and to make quantitative determinations of the enzyme content in rennet solutions (9, 24). Several assay techniques requiring quantitative analysis for a product of the hydrolysis reaction catalyzed by the enzyme to be assayed also have been reported. Substrates that have been used to assay various milk-clotting enzymes are: 1) milk (4), 2) casein (22), 3) hemoglobin (1), 4) synthesized peptides (23, 25), and 5) nonpeptide substrates such as phenyl sulfite (28). Special preparative techniques, which utilize milk and casein as substrates and which allow the reaction product to be detected using absorbance measurement at 217 nm (11), Kjeldahl nitrogen assays (5), or other protein determination techniques [e.g., Lowry et al., (20)], have been used to quantify the product released.

Casein-agar diffusion techniques (3, 14, 15, 19) can be used to determine independently milk-clotting and general proteolytic activity without requiring direct analysis for a product of the hydrolysis reaction. These methods are relatively accurate and sensitive but inconvenient; the casein-agar must be specially handled in controlled temperature areas; each preparation of agar must be calibrated with known enzyme solutions; and assays take up to 48 h to complete.

Historically, the most common method for determining enzymatic activity of proteases has involved measuring the time a milk sample takes to clot after addition of enzyme and relating this time to the quantity of enzyme added. The clotting point can be determined visually when flocculation of milk precipitate begins (2). It can be taken as the time when a rapid increase of viscosity of the milk solution occurs (17, 18) or as the time the milk forms a gel (30). Experimental data indicate that there is a linear relationship between clotting time as defined by these methods and the inverse of the enzyme concentration (16, 17).

Clotting determinations using raw skim milk are not particularly sensitive or accurate as the pH of skim milk (ca. 6.7) is far from the optimum for enzymatic action of rennet and other acid proteases used for clotting milk (13). Even at lower pH, skim milk takes a relatively long time to clot. Furthermore, reproducibility of determinations with skim milk is questionable because "clottability" depends to a degree on the origin and history of the milk sample (33).

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An improved substrate for assay purposes is reconstituted nonfat dry milk (2). Calcium often is added to solutions of this material to promote rapid clotting. Further enhancement of clotting rate and stabilization of the enzyme being assayed is accomplished by lowering the pH of the milk solution to around pH 6.0. Diluting the reconstituted milk to .1 to 5.0% solids provides a solution with a more distinct endpoint for visible coagulation than fully reconstituted milk. The coagulation time in these solutions is substantially less than in skim milk for a given enzyme concentration (12). Very dilute milk solutions (less than .5% solids) can be used for spectrophotometric determination of the coagulation time (21).

The simple "clotting time" assays described are limited as to the minimum enzymatic activity that can be detected accurately. This limit is caused by three factors:

1) Long coagulation times characteristic of low enzyme in the assay solution result in bacterial degradation of milk proteins and changes of clotting properties during assay.

2) Small enzyme concentrations cause the physical flocculation process to proceed so slowly that it is difficult to specify the exact point of coagulation.

3) At low enzyme concentrations the relationship between clotting time and the inverse of enzyme concentration is nonlinear (10). Hence, it is difficult to develop a simple functional equation relating these variables.

Our paper describes a method of increasing sensitivity of the traditional milk-clotting assay. It avoids the aforementioned problems without greatly increasing the inconvenience of the technique. The method involves incubating the test sample in diluted reconstituted skim milk and adding an additional amount of enzyme to clot the sample. Observed clotting time can be related simply to initial amount of enzyme in the sample.

Mathematical Basis of Assay Technique

Flocculation of casein proteins in the presence of calcium ions is highly dependent on the relative amounts of α_s- and κ-casein in the solution (7, 32). The principle action of milk-clotting enzymes is to hydrolyze κ-casein (31), thereby reducing the ratio of κ:α_s in the caseinate system. When this ratio has been reduced sufficiently, a precipitate of calcium caseinate is seen readily if the calcium ion concentration in the solution is high enough. The time at which large agglomerates of flocculated calcium caseinate become visible to the naked eye commonly is called "clotting time".

The enzymatic hydrolysis of κ-casein appears to follow Michaelis-Menten kinetics (11) i.e.

$$\left[\begin{array}{l}\text{rate of } \kappa\text{-casein hydrolysis} \\ = V_m(\kappa)/[K_m + (\kappa)]\end{array}\right] \qquad [1]$$

where (κ) refers to concentration of κ-casein, V_m is the maximum reaction rate, and K_m is the Michaelis constant.

The reaction rate also may be written:

$$d\kappa/dt = [-k_{cat}E_0(\kappa)/[K_m + (\kappa)] \qquad [2]$$

where t is the reaction time and V_m has been replaced by the product of the catalytic constant, k_{cat}, and E_0. The E_0 is the total concentration of enzyme (both bound and free).

For present purposes it is sufficient that the rate of hydrolysis for a fixed substrate concentration be proportional to the total concentration of enzyme (E_0). Hence, equation [2] can be written in the form:

$$d(\kappa)/dt = E_0/f(\kappa) \qquad [3]$$

where for the case described:

$$f(\kappa) = -[K_m + (\kappa)]/k_{cat}(\kappa) \qquad [4]$$

More generally $f(\kappa)$ can take on a number of forms characteristic of steady-state kinetics (6, 26).

If one assumes that no flocculation of casein occurs until a critical amount of κ-casein has been hydrolyzed (7) and that thereafter clotting occurs immediately, the relationship between the clotting time (t_c) and total enzyme concentration can be found by separating variables and integrating equation [3] from t=0; $(\kappa) = (\kappa_0)$ to $t=t_c$; $(\kappa) = (\kappa_c)$.

$$\int_{(\kappa_0)}^{(\kappa_c)} f(\kappa)\, d(\kappa) = \int_0^{t_c} E_0\, dt \qquad [5]$$

where (κ_0) is the initial concentration of

κ-casein and (κ_c) the κ-casein concentration at the moment clotting takes place.

For a constant concentration of enzyme this gives:

$$t_c = \Omega_c/E_0 \quad [6]$$

where:

$$\Omega_c = \int_{(\kappa_0)}^{(\kappa_c)} f(\kappa)\, d(\kappa) \quad [7]$$

Equation [6] predicts that the clotting time is inversely proportional to the total enzyme concentration. This form has been noted by several investigators (17, 29).

The experimentally observed clotting time, t_c(obs), usually is related to the enzyme concentration by an equation of the following form (10, 16):

$$t_c\,(\text{obs}) = \Omega_c/E_0 + t_0 \quad [8]$$

where t_0 is an experimentally determined constant. Verification of the form of equation [8] is shown for a limited range of enzyme concentrations in Figure 1.

This correlation can be explained by assuming that a short time (t_0) must elapse before clotting is observed even after the critical amount of κ-casein has been hydrolyzed, i.e.,

$$t_c\,(\text{obs}) = t_c + t_0 \quad [9]$$

In principle, equation [8] can be used as a basis for measuring the clotting activity of an unknown sample. This is accomplished by measuring the observed clotting time for such a sample and using the parameters t_0 and Ω_c obtained from solutions of known enzyme concentrations to calculate the concentration of the unknown (17). However, this simple procedure becomes impractical for very low enzyme concentrations for the reasons mentioned. The discussion that follows outlines the basis of a method for extending the limit of detection of the standard milk-clotting assay.

If a small amount of enzyme is added to a casein solution first, and if at some time before coagulation another amount of enzyme is added to the milk, the clotting time following the secondary addition of enzyme is less than the clotting time when the two amounts are added simultaneously. In this case the mathematics are:

From t=0 to t=t_1,

$$\int_{(\kappa_0)}^{(\kappa_1)} f(\kappa)d(\kappa) = \int_0^{t_1} E_1\,dt \quad [10]$$

where (κ_1) is the concentration of κ-casein remaining at the time (t_1) when the secondary addition of enzyme is made, and E_1 is the total enzyme concentration (bound plus free) due to the primary addition of enzyme.

From t=t_1 to t=t_c,

$$\int_{(\kappa_1)}^{(\kappa_c)} f(\kappa)d(\kappa) = \int_{t_1}^{t_c} (E_1 + E_2)dt \quad [11]$$

where E_2 is the concentration of enzyme (bound plus free) due to the secondary addition of enzyme.

Addition of equations [10] and [11] gives:

$$\int_{(\kappa_0)}^{(\kappa_c)} f(\kappa)d(\kappa) = \int_0^{t_1} E_1\,dt_1 + \int_{t_1}^{t_c} (E_1 + E_2)dt \quad [12]$$

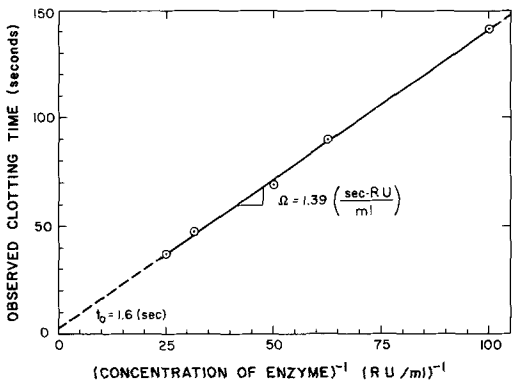

Figure 1. Experimental verification of equation [8]. (Assay solution clotted with rennet extract).

For constant E_1 and E_2 integration yields:

$$\Omega_c = E_1 t_1 + (E_1 + E_2)(t_c - t_1) \quad [13]$$

Again if $t_c = t_c\,(obs) - t_0$:

$$\Omega_c = E_1 t_1 + (E_1 + E_2)(t_c\,(obs) - t_1 - t_0). \quad [14]$$

The term $(t_c(obs) - t_1)$ is the observed clotting time following the secondary enzyme addition. This difference will be termed t_a. Hence:

$$\Omega_c = E_1 t_1 + (E_1 + E_2)(t_a - t_0) \quad [15]$$

Division by $(E_1 + E_2)$ and rearrangement gives:

$$t_a = -\frac{E_1}{E_1 + E_2} t_1 + \frac{\Omega_c}{(E_1 + E_2)} + t_0 \quad [16]$$

Further, from equation [8] the final two terms in equation [16] represent clotting time (t_c') characteristic of an enzyme concentration ($E_1 + E_2$) added to the assay solution at time $t_1 = 0$. Hence,

$$t_c' = \frac{\Omega_c}{(E_1 + E_2)} + t_0 \quad [17]$$

With this substitution equation [16] then becomes:

$$t_a = -\frac{E_1}{E_1 + E_2} t_1 + t_c' \quad [18]$$

This equation predicts that the observed secondary clotting time (t_a) will decrease in a linear fashion as the time when the secondary enzyme is added (t_1) is increased. The slope of a plot of t_a vs. t_1 is related to the relative enzyme concentrations E_1 and E_2 by the equation:

$$\text{slope} = -\frac{E_1}{E_1 + E_2} = -\frac{r}{(r + 1)} \quad [19]$$

where $r = E_1/E_2$.

The intercept t_c' is related to the clotting time observed when a concentration of enzyme E_2 is used to clot the casein solution (t_c^o) by the equation,

$$t_c' = (t_c^o - t_0)\frac{E_2}{E_1 + E_2} + t_0 \quad [20]$$

Figure 2 shows how these variables are related.

The relationship between t_a and t_1 can be used to determine the concentration E_1 in a casein solution. By determining the slope of a plot of t_a vs. t_1, the ratio of the two enzyme concentrations E_1 and E_2 can be estimated because,

$$E_1/E_2 = -\text{slope}/(1 + \text{slope}) \quad [21]$$

If concentration E_2 is known, E_1 can be found.

MATERIALS AND METHODS

Pasteurized, low temperature, nonfat dry milk from Consolidated Badger Co-op, Appleton, WI, was used for all standard experimental test solutions. Rennet extract and Marzyme (single strength) containing 100 rennet units/ml (RU/ml) were commercial milk-clotting preparations obtained from Miles Laboratories. Porcine pepsin (20,000:1) was purchased from Calbiochem as a dry powder. It was dissolved in .1 *M* KH_2PO_4 to make solutions for assays. Other chemicals were reagent grade laboratory stock.

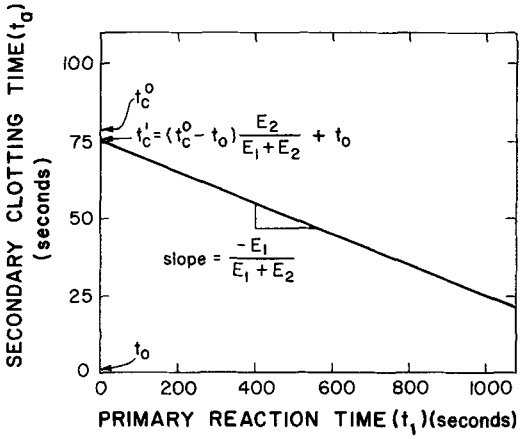

Figure 2. Graphical representation of equation [18].

The assay substrate solution for all experiments was prepared in the following manner unless otherwise noted. For a final substrate volume of 100 ml, .4 ml of 1 N NaOH was added to 50 ml of .1 *M* KH_2PO_4 solution in a 150-ml beaker. Five ml of .1 *M* $CaCl_2$ and 35 ml of distilled, deionized water were mixed in another beaker. The two solutions were combined, and 2.00 g of dry skim milk was added to the combined solution while it was agitated with a magnetic stirrer. After the dry powder was dissolved completely, the solution was transferred to a 100-ml volumetric flask, diluted to the mark with washings from the beaker, and mixed thoroughly by repeated inversions. The substrate solution then was allowed to equilibrate for at least 2 h in the 30°C water bath where clotting times were determined.

Clotting times were determined in basically the same way as in the Berridge method (2) using a Sommer-Matsen rennet tester device. To accommodate 20-ml test tubes instead of the rennet bottles usually employed with the apparatus, the device was modified by #4 cylinder guards added to the rollers. During clotting tests the tubes were rotated at 45 rpm. To obtain more consistent clotting times with smaller random error, five 4-mm glass beads were added to each assay tube. Assay tubes containing glass beads were preincubated in the water bath before use.

For clotting time determination, 1 ml of a milk solution was pipetted into a prewarmed assay tube with a warm pipette. The tube was placed on the rollers of the rennet tester so that the lower half of the tube was submerged under the bath water, and the rotation was started. A predetermined amount of standard enzyme, usually 2×10^{-2} RU per sample, was injected directly into the assay milk just as a stop watch was started. The tube was shaken gently for 3 to 4 s to disperse the enzyme; then it was replaced on the rollers. The watch was stopped at the first sign of coagulation in the tube. The elapsed time for each sample was recorded as its "secondary clotting time".

Enzyme concentrations in an unknown sample were determined as follows. A known amount of an unknown sample was injected into a prewarmed assay solution with a 100-μl syringe, and a primary timer was started to ascertain elapsed time from addition of enzyme. The solution was agitated thoroughly on a vortex mixer for 15 to 30 s, then replaced in the water bath. One-milliliter samples of the assay milk, now containing some enzyme, were transferred to assay tubes containing glass beads as described. At intervals, these solutions were used to make secondary clotting time determinations. The time between mixing of the assay solution with the unknown sample and the start of the secondary clotting time determination is referred to as the "primary reaction time." The relationship between the primary reaction time and secondary clotting time was used to determine the enzyme concentration in the assay solution. This in turn could be related to the enzyme concentration in the unknown solution. A typical sample calculation is described in the Appendix.

RESULTS

Experimental Verification of Equation [18]

The relationship between primary reaction time (t_1) and secondary clotting time (t_a) was tested experimentally for several known primary enzyme concentrations with rennet extract. Figure 3 shows a comparison of clotting data and theoretical predictions of equation [18] for three concentrations (E_1) of enzyme. There were differences between theory and data that cannot be attributed to experimental error. Specifically, this figure indicates that the secondary clotting times obtained at long primary reaction times are greater than those predicted by equation [18]. If these data are

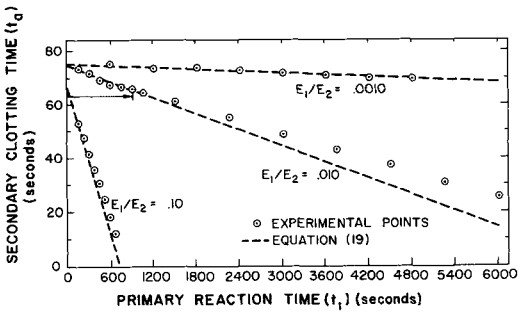

Figure 3. Theoretical and experimental relationship between the primary reaction time and secondary clotting time. (Assay solution clotted with rennet extract. The arrow indicates useful data range for accurate regressions for $E_1/E_2 = .010$.)

used to calculate primary enzyme concentrations via slopes, low concentrations are obtained invariably. This problem also was observed for other clotting enzymes in this study.

Careful examination of a large number of data showed that in the limit as $t_1 \rightarrow 0$ the theory and experimental data coincide. Furthermore, for all practical purposes, theory and experiment are equivalent for a significant range of t_1. If assays are limited to the early portion of the t_1 vs. t_a relationship, slopes that allow accurate determination of the primary enzyme concentration (E_1) can be obtained.

Assays of solutions containing known enzyme concentrations (E_1) were made for rennet extract, pepsin, and "Marzyme". In all cases 2×10^{-2} RU/ml was the concentration of the secondary clotting enzyme (E_2). Concentrations of E_1 were varied to give dilutions (r) of .100, .010, and .001. The results from these assays are in Table 1. The systematic error apparent at the highest primary enzyme concentration (r=.100) is due to inability of data to be taken at sufficiently small t_1. Figure 4 shows a more complete set of data for rennet extract.

Use of Technique for Determination of Enzyme in Milk and Whey

A sample of reconstituted skim milk (10% solids, .01 *M* added $CaCl_2$, pH 6.6) was coagulated by adding 2×10^{-2} RU of rennet extract per milliliter of milk. After enzyme addition, but before coagulation, 100 μl of this milk was added to 9.9 ml of assay solution. The enzyme concentration was determined by the methods described. The amount of enzyme from this assay technique was $2.04 \pm .14 \times 10^{-2}$ RU/ml (see Table 2).

Fifty minutes after rennet addition, the milk gel that formed was cut repeatedly with a spatula, and the resulting slurry was centrifuged at 900 × *g* for 10 min to separate whey and curd. The whey was analyzed for enzyme as described and contained $1.75 \pm .08 \times 10^{-2}$ RU/ml. For the distribution of whey and curd (37.5 g whey, 12.5 g curd), this corresponds to 66% recovery of the enzyme in the whey fraction, in agreement with observations of Holmes et al. (15).

An amount of enzyme corresponding to a concentration of 2.00×10^{-2} RU/ml was added to a portion of this whey, and this sample was analyzed for enzyme content. The new concentration of enzyme, including the added enzyme, was $3.86 \pm .14 \times 10^{-2}$ RU/ml. Thus, the amount added was determined experimentally as $2.11 \pm .16 \times 10^{-2}$ RU/ml, indicating that the method can determine enzyme accurately in whey solutions. Table 2 summarizes these findings.

Assay of Enzyme in Curd

An attempt was made to assay directly for enzyme contained in curd by adding frozen and

TABLE 1. Determination of various enzymes.

Enzyme	Dilution factor[1] r	Calculated slope b	Slope standard deviation	% Recovery	SD	n+	m+
Rennet extract	.1000	−.08179	.00115	89.1	1.2%	3	8
Porcine pepsin	.1000	−.08429	.00110	92.1	1.1%	3	8
Marzyme	.1000	−.07859	.00082	85.3	.8%	3	8
Rennet extract	.0100	−.01025	.00102	103.5	10%	0	12
Porcine pepsin	.0100	−.00956	.00118	96.5	12%	0	12
Marzyme	.0100	−.00974	.00120	98.4	12%	0	12
Rennet extract	.0010	−.00098	.00007	98	7%	8	24
Porcine pepsin	.0010	−.00106	.00011	106	11%	8	24
Marzyme	.0010	−.00104	.00008	104	8%	8	24

[1]Dilution factor = concentration of primary enzyme/concentration of secondary enzyme = E_1/E_2 where $E_2 = 2 \times 10^{-2}$ rennet units/ml in all runs, n+ = number of intercept points used in the determination, and m+ = number of nonintercept points used in the determination.

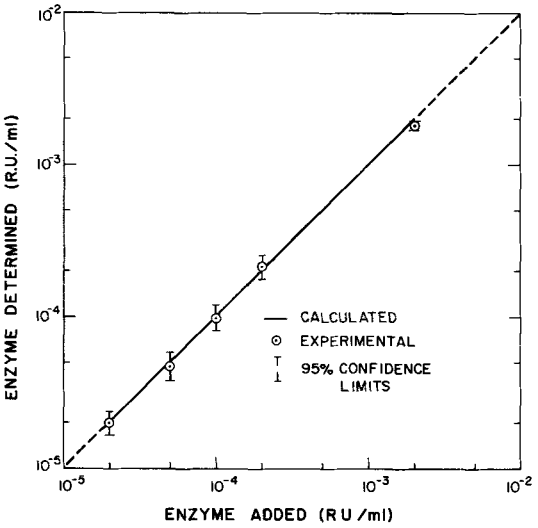

Figure 4. Use of the assay technique to determine the enzyme content of standard solutions containing rennet extract.

ground curd to an assay solution. This technique yielded an assay of only 1.88×10^{-2} RU/g of curd compared to an expected 2.75×10^{-2} RU/g. This result is probably due to an incomplete release of the enzyme from the curd matrix.

Other Assay Solutions

The assay technique described should be expected to work with many assay solutions. A solution containing 2% wt/vol dry skim milk solids, .0125 *M* $CaCl_2$, and .04 *M* HCl was used for some determinations on known samples. This assay solution produced satisfactory results for rennet extract and Marzyme determinations but slightly low results for pepsin determinations. This phenomenon was not investigated thoroughly or resolved, but there is some indication that the low pepsin determinations may have been due to nonspecific adsorption of the pepsin on the glass in the assay system.

DISCUSSION OF RESULTS

The experimentally observed result that the relationship between primary reaction time and secondary clotting time is not linear over the entire primary reaction range is unfortunate because it limits precision of this technique and makes it less convenient to use. Computer simulation studies have indicated that the deviation from theory seen in the experimental results could be caused by a nonenzymatic reaction, which must occur before clotting can be observed. Such a reaction likely involves hydrolysis products of κ-casein. Because the physical flocculation of micelles involves this nonenzymatic reaction, experimental observations can be explained partially in these terms.

However, even with the inconvenience caused by this nonlinear relationship, this technique still is used more easily and more rapidly than other techniques. Enzyme concentrations as small as 2×10^{-5} RU/ml in assay solutions are determined easily to ± 10% precision in less than 2 h. This can be compared with a conventional assay limit of 10^{-4} RU/ml (which takes up to 48 h) in

TABLE 2. Determination of enzyme in milk and whey.

Sample	Amount added $\times 10^{-2}$ (RU[1]/ml)	Amount determined $\times 10^{-2}$ (RU/ml)		% Recovery	
			SD		SD
Milk	2.00	2.04	.14	102	7
Whey	...	1.75	.08	88	4
Whey + 2.00×10^{-2} RU/ml	3.75 ± .08	3.86	.14	103	4
Difference in whey samples	2.00	2.11	.16	105.5	8

[1] Rennet units.

[2] Concentration of enzyme in whey relative to that in milk.

casein agar diffusion tests. Even considering that the unknown usually is diluted into the assay milk, it should be possible to assay solutions containing 10^{-4} RU in a much shorter time. The technique should be particularly useful in assaying for enzyme in milk solutions that have been passed through immobilized enzyme columns. A continuous decrease of secondary clotting time after the solution exits the column would be an indication that there is soluble enzyme in the solution. The rate of decrease could be used to estimate the amount of soluble enzyme.

It should be possible to use this general procedure in assaying for other enzymes with other assay solutions. In any enzymatic reaction system, the basic mathematical technique described herein can be used. For example, the enzymatic hydrolysis of benzoyl arginine ethylester by trypsin results in the release of a hydrogen ion. From a stock solution, the total conversion can be indicated by the change of pH that results from the reaction. By adding an indicator such as phenophthalein to the reaction mixture, a given extent of reaction can be indicated. The color change reaction in this case would be analogous to the clotting reaction.

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APPENDIX

Typical Example

Three replicates of an assay solution containing 2×10^{-5} RU were prepared by 5 μl of standard chymosin (.4 RU/ml) injected into each of three test tubes containing 10 ml of assay milk. One tube containing assay solution but no added enzyme was used as a control and for intercept determinations (see following discussion). All tubes were preincubated in a 30°C water bath.

After the 10-ml tubes were agitated thoroughly, 1 ml samples of the solution were transferred with a warm pipet into prewarmed test tubes containing glass beads. At 10-min intervals an additional 2×10^{-2} RU/ml was added to these 1-ml subsamples by 5 μl of 4.0 RU/ml enzyme standard solution injected into the test tubes while they were rotated in the bath. Clotting times were determined with a stopwatch by the exact time measured from the secondary injection to the onset of visible flocculation. The experimental results for a control and three replicates are in Table 1-A.

A significant improvement of precision of the determinations was obtained by inclusion in the regression analysis of experimental data from the secondary clotting times of control samples. Strictly speaking, these data do not belong to the regression set because for these determinations the total concentration of enzyme is only (E_2) rather than ($E_1 + E_2$) as for the regular regression points.

The secondary clotting time measures from control samples however, can be adjusted to represent intercepts ($t_1 = 0$) for unknown determinations by suitable modification of the experimental measures. As was pointed out, the intercept of the t_1 vs. t_a plot equals the clotting

TABLE 1-A. Experiment data for the example.

Primary reaction time (t_1) (min)	Secondary clotting time (t_a) (s)			
		Replicates		
	Control	1	2	3
10	75.65	74.94	74.05	74.31
20	76.36	72.76	73.36	72.50
30	75.25	72.99	73.08	72.41
40	75.72	71.87	72.97	72.64
50	74.44	72.55	71.34	71.59
60	74.11	71.39	72.48	71.11
70	74.41	70.42	70.52	69.97
80	73.81	69.33	69.93	71.10

[1]The data from replicates 1, 2, and 3 were pooled and fit to equation [18]. They were analyzed in terms of standard linear regression formulae to determine values for the slope (b), intercept (t'_c), standard deviation (S_{xy}), and standard deviation of the slope (S_b) (33). Results are in Table 2-A.

TABLE 2-A. Regression analysis.

	Replicates only	Replicates plus intercept
Slope (b)	−.00091	−.00098
Slope standard deviation S_b	.00009	.00007
Data standard deviation S_{xy}	.58	.67
Intercept	74.51	74.75
Number of regression pts.	24	32

time of a control sample with a concentration of enzyme equal to $(E_1 + E_2)$. The clotting time of a control sample containing a concentration of enzyme E_2 is (t_c) and is related to that of a sample containing a concentration $(E_1 + E_2)$ by equation [20]. If t_0 in equation [20] is set equal to zero, then equation [20] becomes:

$$t_c' = t_c^\circ \frac{E_2}{E_1 + E_2} \qquad [A\text{-}1]$$

When data are fit to equation [18], the estimated slope of the regression line (b) is given by:

$$b = -E_1/(E_1 + E_2) \qquad [A\text{-}2]$$

so that:

$$1 + b = E_2/(E_1 + E_2) \qquad [A\text{-}3]$$

Equation (A-1) then can be written as:

$$t_c' = t_c^\circ (1 + b) \qquad [A\text{-}4]$$

For a set of t_{cj}° for the control sample, a corresponding set t_{cj}' of intercepts for the assay sample can be calculated if b is known. The calculated b depends on the intercepts ($t_1 = 0$, $t_a = t_{cj}'$) used in the calculations. This fact suggests that an iterative procedure to find b is appropriate.

A good first estimate of b, (b_0), can be calculated from equation 18 without including any intercepts. This will allow calculation of a first set of intercept values, $t_c'^{(1)}{}_j$ according to:

$$t_c'^{(1)}{}_j = t_{c\,j}^\circ (1 + b_0) \qquad [A\text{-}5]$$

An improved estimate of b, $b_{(1)}$ then can be calculated by $t_c'^{(1)}{}_j$ included in a second regression calculation by equation [18]. A revised estimate of t_c, $t_c^{(2)}$ can be made by using the new estimate of b, $b_{(1)}$ according to:

$$t_c'^{(2)}{}_j = t_{c\,j}^\circ (1 + b_{(1)}) \qquad [A\text{-}6]$$

and so on.

This iterative procedure will converge to a constant b, usually in 2 to 3 iterations. This provides the most precise estimate of b that can be obtained with the data.

Regression of the data (including the eight intercepts in Table 1-A) used this procedure. The regression results for such a calculation are in Table 2-A.

A simple calculation allows one to determine the enzyme concentration (E_1) from slope data. In the case described here $E_1 = 1.96 \pm .14 \times 10^{-5}$ RU/ml as opposed to the known 2.00×10^{-5} RU/ml.